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Autoantibodies produce pain in Complex Regional Pain Syndrome by sensitizing nociceptors

Ulku Cuhadar[1], Clive Gentry[1], Nisha Vastani[1], Serena Sensi[2], Stuart Bevan[1], Andreas Goebel[2†] and David Andersson[1]*[†]

[1]Wolfson Centre for Age-Related Diseases, Institute of Psychiatry, Psychology & Neuroscience, King's College London, SE1 1UL, London, United Kingdom.

[2]Pain Research Institute, University of Liverpool, and Walton Centre NHS Foundation Trust, L9 7AL, United Kingdom.

†These authors contributed equally to this manuscript.

*To whom correspondence should be addressed:

David Andersson, Wolfson CARD Hodgkin Building, Guy's Campus, London, SE1 1UL, United Kingdom

+442078486141

david.andersson@kcl.ac.uk.

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Abstract

Complex regional pain syndrome (CRPS) is a post-traumatic pain condition with an incompletely understood pathophysiological basis. Here, we have examined the cellular basis of pain in CRPS using behavioral and electrophysiological methods in mice treated with IgG from CRPS patients, in combination with a paw incision. Mice were subjected to a hind paw skin-muscle incision alone, or in combination with administration of IgG purified from either healthy control subjects (HC) or patients with persistent CRPS. Nociceptive function was examined behaviorally *in vivo*, and electrophysiologically *in vitro* using skin-nerve preparations to study the major classes of mechanosensitive single units. Administration of IgG from CRPS patients exacerbated and prolonged the post-surgical hypersensitivity to noxious mechanical, cold and heat stimulation, but did not influence tactile sensitivity following a paw incision. Studies of IgG preparations pooled from patient cohorts (n=26-27) show that pathological autoantibodies are present in the wider population of patients with persistent CRPS, and that patients with more severe pain have higher effective autoantibody titres than patients with moderate pain intensity. Electrophysiological investigation of skin-nerve preparations from mice treated with CRPS IgG from a single patient identified both a significantly increased evoked impulse activity in A- and C-nociceptors, and an increased spontaneous impulse rate in the intact saphenous nerve. Our results show that painful hypersensitivity in persistent CRPS is maintained by autoantibodies, which act by sensitizing A- and C-nociceptors.

Keywords: Complex Regional Pain Syndromes; nociceptors; Passive transfer; IgG; mice

Introduction

Complex regional pain syndrome (CRPS) is a post-traumatic condition typically confined to a single limb. CRPS is characterized by pain of disproportionate intensity and duration compared to that expected from the clinical time course of the initial injury or trauma, but the etiology and pathophysiological basis of CRPS are incompletely understood[6,22,37,39,53]. Distal limb fractures are the most common triggers of CRPS, but the severity of CRPS is independent of the initiating trauma, and apparently trivial insults can lead to CRPS[43,61]. The affected limb typically exhibits several characteristic abnormalities, including increased sensitivity to both normally innocuous and painful stimuli, skin discoloration, fluctuating temperature asymmetry (compared to the uninjured limb), swelling, sudomotor alterations, and trophic changes to skin, hair and nails. Pain is, however, the dominant symptom and CRPS is not associated with tissue destruction[9,53]. No diagnostic tests exists for CRPS, and patients are diagnosed based on clinical symptoms and signs[10,23,30]. Most patients recover spontaneously, but 15-20% of patients develop persistent severe pain, which may last for life and is associated with an exceptionally low quality of life, even when compared to other chronic pain conditions[40,59]. The characteristic autonomic abnormalities seen early in the course of CRPS typically normalize in patients with long-standing disease, whereas pain, as well as sensory and motor abnormalities may remain. This has led to suggestions that persistent CRPS is sustained by abnormal central nervous system plasticity[46].

Earlier investigations have demonstrated that administration of IgG from patients with persistent CRPS (>1 year duration) to mice reduced spontaneous rearing in freely moving mice[28]. When IgG transfer was combined with a paw incision as an experimental insult, a transiently enhanced postsurgical swelling and mechanical hypersensitivity in the injured paw was observed[27].

Later studies demonstrated that immunoglobulins of other isotypes were inactive, since transfer of IgG-depleted serum was without effect[55]. These results indicate that IgG autoantibodies are central for the genesis and maintenance of pain and painful hypersensitivities in persistent CRPS. Importantly, the underlying neuronal mechanisms and sites of action responsible for pain have not been identified.

In this study, we have performed a detailed behavioral and electrophysiological investigation of painful sensory abnormalities in the passive transfer-trauma model of CRPS (tCRPS[55]) and identified peripheral sensitization of nociceptors as a major mechanism by which autoantibodies produce pain in CRPS.

Materials and Methods

Study Design

We examined the impact of a paw incision in combination with administration of serum IgG purified from healthy control subjects or CRPS patients on mouse nociception both *in vivo and ex vivo*.

Research subjects

The main immunoglobulin donor was a 40 year old female who had CRPS of 9 years' duration in a lower limb and a high average pain (9-10/10 on a 11-point numeric rating scale (NRS) with 10=the worst pain imaginable) and had been offered clinical plasma exchange treatment on compassionate grounds[2,26]. She had signs in all four Budapest diagnostic categories[30] and alternative causes for her pain had been excluded by a pain specialist, a consultant rheumatologist and a consultant neurologist. This patient clinically exhibited strong pain to mild pressure over the painful area (*i.e.* mechanical hyperalgesia, the most common sensory abnormality in persistent CRPS[23,32]), whereas she had little or no pain to light touch; she reported that ambient temperatures below room temperature and also temperatures above approximately 24°C would increase her pain. Waste plasma from the first exchange treatment was secured with her consent and was stored frozen – the local Ethics committee confirmed that the use of human waste tissue did not require ethical permission. She received three plasma exchange treatments over 5 days through a central venous line, but unfortunately the treatment had to be ceased thereafter as a consequence of clotting around the tip of the line. She reported no pain relief following this treatment; in line with earlier observations indicating that 7-8 exchange treatments over 3 weeks may be required before meaningful pain relief occurs in this condition[2,26,49].

For experiments on pooled IgG, serum samples were randomly selected from an anonymized list of 111 stored frozen samples (-80°C) and stratified according to either moderate (NRS 5.0-7.0, 26 patients) or high (NRS 7.3-9, 27 patients) baseline pain intensity,. The serum samples were provided by participants in the recently-completed LIPS-trial[25]. The LIPS study-inclusion criteria were patients with persistent CRPS of between 1 and 5 years' duration, fulfilling international research criteria for the diagnosis of CRPS[30], who had an average pain intensity at baseline of at least 5/10. Ethical permission and individual consent for the use of these sera for the purpose of autoantibody research is available (12/EE/0164, East of England).

IgG purification

IgG was purified as described previously[55], using protein G beads (Sigma-Aldrich, Gillingham, UK). Briefly, serum was diluted 1:3 with Hartmann's solution, passed through a protein G column, and the bound IgG was eluted using 100 mM glycine pH 2.3, the pH was adjusted to 7.4 using 1 M Tris pH 8. The preparation was then dialyzed overnight at 4°C in Hartmann's solution using a 10 kDa dialysis membrane (Fisher Scientific, Loughborough, UK). The concentration of IgG present after dialysis was determined using a modified Lowry assay (DC protein assay, BioRad, Hemel Hempstead, UK) and adjusted by dilution with Hartmann's solution or by dialysis against a sucrose solution (Sigma-Aldrich). Finally, the IgG solution was sterile filtered using syringe-driven 0.2 µm filter units (Millipore, Watford, UK), stored at 4°C and used within 3 months.

Animals

Surgical procedures and behavioral experiments were carried out according to the U.K. Home Office Animal Procedures (1986) Act. All procedures were approved by the King's College London Animal Welfare and Ethical Review Body and conducted under UK Home Office Project Licenses PPL 70/7510 and PF0C9D185. Experiments were performed on female C57Bl/6J mice (8– 10 weeks old) obtained from Envigo UK Ltd., Bicester, UK, housed in a temperature-controlled environment with a 12h light/dark cycle and with access to food and water *ad libitum*. Mice were injected intraperitoneally with Hartmann's solution containing 0.8-16mg of IgG from either healthy control subjects or CRPS patients on 4 consecutive days, or as indicated.

Plantar Incision

On the day of the second IgG injection, a 5 mm long midline incision was made through the plantar skin fascia starting 2 mm from the heel and extending towards the toes, using aseptic techniques. The underlying plantar muscle was elevated with curved forceps and incised longitudinally, leaving the muscle origin and insertion intact[8]. The skin incision was then closed using sutures (Mersilk 7/0) and the animals were housed on paper bedding for the first 3 days post-surgery.

Behavioral studies

Before any nociceptive testing, mice were kept in their holding cages to acclimatize (10-15 min) to the experimental room. Mice were randomized between cages and the experimenter blinded to their treatment.

The Randall-Selitto paw-pressure test was performed using an Analgesymeter (Ugo-Basile, Italy). The experimenter lightly restrained the mouse and applied a constantly increasing pressure stimulus to the dorsal surface of the hind paw using a blunt conical probe. The nociceptive threshold was defined as the force in grams at which the mouse withdrew its paw[44]. A force cut-off value of 150 g was used to avoid tissue injury.

Tactile sensitivity was assessed using von Frey filaments (0.008-2 g) according to Chaplan's up-down method[12]. Animals were placed in a Perspex chamber with a metal grid floor allowing access to their plantar surface and allowed to acclimatize prior to the start of the experiment. The von Frey filaments were applied to the plantar surface of the hind paw with enough force to allow the filament to bend, and held static for approximately 2-3 s. The stimulus was repeated up to 5 times at intervals of several seconds, allowing for resolution of any behavioral responses to previous stimuli. A positive response was noted if the paw was sharply withdrawn in response to filament application or if the mouse flinched upon removal of the filament. Any movement of the mouse, such as walking or grooming, was deemed an unclear response, and in such cases the stimulus was repeated. If no response was noted a higher force hair was tested and the filament producing a positive response recorded as the threshold.

Thermal sensitivity was assessed using a hot- and cold-plate (Ugo Basile, Milan). Paw withdrawal latencies were determined with the plate set at a chosen temperature (50 °C for hot-plate and 10 °C for cold-plate tests). The animals were lightly restrained (scruffed) and each hind paw in turn was placed onto the surface of the plate[1,21]. The latency to withdrawal of the paw was taken as the endpoint and recorded for the ipsilateral and the contralateral paw. A maximum cut-off of 30 seconds was used for each paw.

Human IgG ELISA

Human IgG ELISA Kit ab195215 (Abcam, Cambridge, UK) was used to measure the transferred human-IgG concentration in mouse plasma. Plasma was prepared from whole blood using heparin treated tubes and stored at -20°C until use according to the provided instructions from the manufacturer.

Skin-nerve recording

Mice were killed by cervical dislocation and the hind paw was shaved prior to dissection of the isolated skin-nerve preparation. The saphenous nerve and the shaved skin of the hind limb were placed in a recording chamber at 32 °C. The chamber was perfused with a gassed (95% O_2 and 5% CO_2) prewarmed synthetic interstitial fluid (SIF): 108mM NaCl, 3.5mM KCl, 0.7mM $MgSO_4$, 26.2mM $NaCO_3$, 1.65mM NaH_2PO_4, 1.53mM $CaCl_2$, 9.6mM sodium gluconate, 5.55mM glucose and 7.6mM sucrose. The skin was placed inside up (corium side up) and pinned down using insect pins (0.2 mm diameter) in the organ bath to allow access to the receptive fields. The saphenous nerve was fed through a small gap from the organ bath to an adjacent recording chamber and placed on a mirror platform. The desheathed saphenous nerve was covered with paraffin oil for electrical isolation and either whole nerve or dissected fine nerve filaments were placed on a fine gold wire-recording electrode[45,63].

Conduction velocity

The saphenous nerve was divided into progressively thinner filaments until a single unit could be isolated in response to mechanical stimulation of the receptive field with a glass rod. Identified units were electrically stimulated with brief, 1ms pulses (Digitimer DS2, Digitimer Ltd, Welwyn Garden City, UK) and the action potential latency was used to determine the conduction velocity and to categorize units as Aβ (>10m/s), Aδ (1.2-10m/s) or C-fibers (<1.2m/s).

Mechanical stimulation

A computer-controlled stimulating probe, equipped with a force transducer, was used to deliver mechanical stimuli to the most sensitive spot of a receptive field (Axolent UG, Erlangen, Germany). The mechanical threshold for each unit was then determined iteratively by applying a series of 2s mechanical force steps. The mechanical threshold was determined as the force required to elicit at least two action potentials. The adaptation properties of single units were characterized by stimulation with 10s force step challenges in the range 0.5-20g with a 2 min recovery period between challenges. Aβ units that fired briefly during the application and removal of the force were considered rapidly adapting (RA), and those that sustained firing throughout the challenge were classified as slowly adapting (SA). Low-threshold Aδ units with a transient action potential discharge pattern (on and off) were identified as D-hair fibers. The coding properties of Aδ and C-fibers were additionally examined using a series of force ramp stimuli (0.5-20g, 15s duration, with a 2 min recovery period between challenges). Recording and analysis were done using Spike 2 (Cambridge Electronic Design, Cambridge, UK).

Statistical analysis

Data are expressed as mean ± SEM of the number of animals or nerve fibers indicated (n). Behavioral data were analyzed using unpaired t-test, Mann-Whitney U test (when Levene's test of equality of variances $p<0.05$) or ANOVA (followed by Tukey's, Dunnett's or Holm-Sidak's) as appropriate. Statistical tests were performed in SPSS 24 (IBM), Statistica (Tibco), SigmaPlot 14, or Excel 2016 (Microsoft).

Results

Passive transfer of CRPS pain (tCRPS)

We examined the behavioral phenotype of tCRPS by comparing mice subjected to a paw incision alone with mice that additionally received IgG from a CRPS patient or a healthy control subject (HC) on 4 consecutive days (8mg by intraperitoneal injection), starting the day before paw incision. In these experiments, we used IgG from a patient who had CRPS with high average pain scores (9-10 on a 11 point numerical rating scale), since this may indicate a high titre of pathogenic autoantibodies[16]. Following the paw incision, mice in all treatment groups developed hypersensitivity to noxious mechanical stimulation in the paw pressure test (Fig. 1, A and B), and to tactile stimulation with von Frey filaments (Fig. 1, C and D). Mice that had been subjected to a paw incision alone or in combination with IgG from control subjects recovered fully from the postsurgical mechanical hyperalgesia measured by paw pressure thresholds within three days, whereas tCRPS mice retained the maximal level of hypersensitivity without signs of recovery during the assessment period. In contrast, all three treatment groups recovered from the postoperative tactile allodynia assessed with von Frey filaments at a similar rate. The mechanical sensitivities of the uninjured, contralateral paws were unaffected, or minimally affected (Fig. 1, B and D), confirming that both circulating CRPS IgG and trauma are required for the onset of tCRPS.

The donor-patient reported increased sensitivities to cold and heat, as is common in CRPS [23,32], and we therefore also assessed thermal nociception using cold and hot plate assays. Mice treated with CRPS IgG progressively developed significant cold and heat hypersensitivities, associated with a significant contralateral sensitization, whereas the other treatment groups displayed less marked changes and recovered quickly (Fig. 1E-H). Our observations indicate that CRPS IgG administration dramatically exacerbates and prolongs painful hypersensitivities produced by a minor surgical trauma and accurately translates the sensory abnormalities observed in the donor patient to mice.

Dose-dependence of CRPS IgG passive transfer

To determine the dose requirements for successful passive transfer of sensory abnormalities, we administered patient IgG at doses between 0.8 to 8 mg on 4 consecutive days, with one group of mice only receiving two injections of 8 mg. Administration of 8 mg CRPS IgG only on the first two days was without effect compared to HC IgG (Fig. 2AB). Lower doses of CRPS-IgG (4x0.8mg, 4x4mg) induced no or only minimal detectable abnormalities to mechanical stimuli (Fig. 2A) whereas administration of 4mg CRPS IgG per day produced an intermediate level of cold hypersensitivity for the duration of the experiment (Fig. 2B). We monitored the behavioral profile of all groups for a week after the incision but extended this to two weeks for mice treated with 4x8mg CRPS IgG (and the corresponding HC group), since this was the only treatment group that displayed mechanical hypersensitivity. In the 4x8 mg group, hypersensitivities to noxious mechanical (Fig. 2A) and cold stimulation (Fig. 2B) were maintained for at least two weeks. The contralateral paw displayed a transiently and marginally increased sensitivity to noxious cold or mechanical stimulation in the 4x8mg group, which resolved by day 6 (Fig. 2C, D).

To determine whether the circulating human IgG concentration was correlated with the observed behavioral phenotype, we measured the plasma concentration of human IgG by ELISA on days 3, 8 and 13 after incision in mice treated with HC-IgG or CRPS-IgG. On the day of the last injection (day 3) the plasma concentration of human IgG was 19±9 mg/ml, similar to that normally found in human subjects (6-16mg/ml). At later time points (day 8 and 13) the circulating human IgG concentration was negligible (2.3±0.3 and 2.6±0.3 ng/ml). Collectively, these results show that induction of the tCRPS sensory phenotype requires doses similar to those used for passive transfer of other autoantibody mediated neurological conditions, such as *myasthenia gravis*[14,56,57].

The tCRPS phenotype reflects the donor patients' pain intensities

To determine whether the sensory phenotype observed in tCRPS mice is related to the donor-patients' pain intensities, we next compared the effects of IgG preparations pooled from patients from the LIPS trial[25]. Patients were stratified according to their baseline pain intensities within moderate (n=26; 6.0±0.8 mean±SD; range 5.0-7.0) and high (n=27; 8.3±0.4; range 7.3-9.5; table 1) ranges on a 11-point numerical rating scale (NRS) [7]. Pooled IgG (4x8mg) from patients with severe pain, but not from patients with moderate pain, produced significant mechanical hypersensitivity 7 days after the incision, when the postsurgical hypersensitivity seen in mice that were only subjected to a paw incision had recovered fully (Fig. 3A, see Fig. 2A). This observation indicates that the degree of mechanical hyperalgesia in tCRPS mice reflects the donor patients' spontaneous pain intensities. The different effects of IgG from patients with moderate or high pain may further suggest that target heterogeneity or autoantibody titre influences symptom severity as in other autoantibody-medicated painful conditions[33]. Alternatively, autoantibodies may play no role in the group of patients with only moderate pain. To investigate this latter possibility further, we examined the effect of a larger dose (16mg/day, Fig. 3B) of pooled IgG from patients with moderate pain intensity and found that this larger dose produced significant hypersensitivity compared to HC IgG (16mg/day). Interestingly, this effect was more transient than that observed in mice treated with IgG (8mg) from patients with higher pain intensity. As the contributions of IgG from individual patients were diluted in these pooled IgG samples (26-27 patients), our results strongly suggest that autoantibodies are universally responsible for maintaining pain in patients with persistent CRPS. The time required for mice to recover from tCRPS appear variable, with full recovery observed within 2 weeks for the pooled IgG preparations (Fig. 3B), but only a partial recovery with the main patient donor over the same period (Fig. 2).

CRPS IgG induces spontaneous impulse generation in skin-saphenous nerve preparations

Ectopic sensory afferent impulse discharge generates spontaneous pain and paraesthesias in rodents and human patients[50,51,60]. Persistent spontaneous pain is a hallmark of CRPS, and we therefore recorded the activity of the intact saphenous nerve, before splitting the nerve into thin filaments for studies of single units described below. In this configuration, IgG from the main patient donor significantly increased the spontaneous ongoing impulse rate (7.1±2.4Hz) compared to preparations from naïve (0.9±0.4Hz), incision only (1.4±0.4Hz) and HC IgG (1.4±0.5Hz) treated mice ($p<0.01$, Kruskal-Wallis test, Fig. 4, preparations harvested 3 days after incision). This demonstration of enhanced ectopic activity in an *in vitro* preparation isolated from the central nervous system suggests that CRPS-IgG exerts a peripheral pathogenic and proalgesic effect.

Electrophysiological investigations of mechanosensitive sensory afferents

Mechanical hypersensitivity is an almost ubiquitous sensory abnormality in patients with persistent CRPS[32], and a prominent characteristic of the tCRPS phenotype (Figs. 1-3), which suggests that CRPS IgG alters the function of mechanosensitive nociceptors. Therefore, we examined the function of mechanosensitive single units in skin-saphenous nerve preparations. This approach allowed us to quantify the afferent action potential responses evoked by mechanical stimulation of the receptive fields in their intact anatomical and physiological context. We determined the mechanical activation threshold, conduction velocity and temporal response profiles of Aδ- and C-mechanonociceptors (AM and CM), as well as of the low threshold mechanosensitive fiber types, Aβ- (RA and SA), and D-hair Aδ-fibers. We did not observe a significant spontaneous impulse activity in the recorded single units.

A-mechanonociceptors

Aδ-mechanonociceptor (AM) fibers in preparations from tCRPS mice displayed a modestly reduced mechanical force threshold for activation (Levene's $p<0.05$, Mann-Whitney $p=0.056$, Fig. 5A), but an unaltered conduction velocity compared to preparations from mice only subjected to a paw incision (Table 2). Application of mechanical step- or ramp-shaped force stimuli (from 0.5 to 20g, applied for a duration of 10 and 15s, respectively) demonstrated that AM fibers in tCRPS preparations responded with a higher impulse rate throughout the range of forces used (Fig. 5B-G). Analysis of the impulse pattern evoked by a 5g force step challenge, revealed that tCRPS AM fibers responded with a markedly increased impulse rate, but with a very similar temporal profile compared to that observed in preparations from mice that were not treated with IgG (Fig. 5D, F). AM-fibers encode increasing mechanical force with a corresponding linear increase in impulse frequency[36], and our results show that this force-impulse relationship in response to ramp stimuli is significantly steeper in AM fibers from tCRPS mice than in non-IgG treated mice (Fig. 5E, G). The combination of a steeper force-impulse rate relationship, and a relatively minor force threshold reduction suggests that both transduction and excitability processes are sensitized by CRPS IgG in tCRPS mice (Fig. 5A, E). In keeping with the steeper force-impulse relationship observed in tCRPS AM fibers, the maximal impulse discharge rates evoked by either a 20g force step (Fig. 5H) or a force ramp to 20g (Fig. 5I) were increased significantly, compared to AM fibers in preparations from mice that were only subjected to a paw incision.

C-mechanonociceptors

The mechanical threshold for activation of C-mechano-nociceptor (CM) fibers was significantly reduced in preparations from tCRPS mice, compared to preparations from mice that had only undergone a paw incision (Fig. 6A). The force thresholds of single units in the control group were distributed through the range 0-7g, whereas all units in tCRPS preparations responded at forces below 3g. The C-fiber conduction velocity did not differ significantly between the treatment groups (table 2). Analysis of the response pattern evoked by a series of force steps and ramps identified a significantly increased impulse rate in tCRPS CM fibers at the highest forces used (Fig. 6B-G, Levene's, $p<0.05$, Mann-Whitney, $p<0.05$). The temporal distribution of action potentials during stimulation with mechanical force appeared unchanged, which is similar to our observations in AM-fibers (Fig. 6F, G). The maximal impulse discharge rate was increased in preparations from tCRPS mice compared to paw incision alone (Fig. 6H, I).

Low threshold mechanosensitive A-fibers

We assessed the functional impact of tCRPS on the major classes of low-threshold mechanosensitive afferent fibers; slowly adapting Aβ- (SA), rapidly adapting Aβ- (RA) and D-hair (DH) Aδ-fibers. Consistent with the absence of tactile allodynia in tCRPS mice *in vivo* (see Fig. 1C), the conduction velocities and mechanical force activation thresholds of SA, RA and DH fibers were indistinguishable in preparations from the two treatment groups (Table 2, Fig. 7A-F). In good agreement with earlier characterizations [36], mechanically evoked responses in control RA and SA units did not encode forces above 5g with an increasing number of action potentials. The impulse discharge rates of RA and SA fibers did not differ between treatment groups (Fig. 7B, D). Similarly, we could not distinguish between the numbers of action potentials generated in response to force steps in DH fibers in preparations from tCRPS and incision only mice (Fig. 7E, F).

Discussion

Here we demonstrate that administration of IgG from patients with persistent CRPS, in combination with a minor experimental insult, transfers persistent mechanical, as well as thermal sensory abnormalities from donor patients to mice ('tCRPS'). We further show that the degree of transferred sensory abnormalities correlates both with the transferred IgG dose, and the respective donor-patients' subjective pain intensities. Electrophysiological investigations of skin-saphenous nerve preparations from mice treated with IgG from a typical patient-donor demonstrate a markedly increased stimulus-evoked discharge rate in A- and C-mechanonociceptor single units, accompanied by a reduction in the mechanical response threshold. In contrast, low-threshold A-fibers were unaffected by CRPS patient IgG. The single nerve fiber properties thus reflect both this donor patient's clinical experience and the transferred behavioral phenotype observed in mice. We studied saphenous single units with receptive fields in the uninjured dorsal hind paw skin, rather than the incised plantar skin. The functional abnormalities observed in these nociceptors are thus not directly explained by the postsurgical hypersensitivity seen in sural and tibial single units innervating the incised plantar skin [3,8], but are consistent with the more generalized regional pain seen in patients who develop CRPS after injury[6,53].

After administration, IgG distributes into the interstitial fluid of tissues, and its pharmacokinetic behavior can typically be described using a two-compartment model, with a rapid, first-order elimination from circulation, and a slower second-order component clearing IgG from tissues [48]. The behavioral phenotype of tCRPS is stable for at least two weeks, a time course that is consistent with the observed terminal half-life of human IgG in mice[48,58].

Although most of the results presented here were collected using IgG from a single patient, IgG pooled from 27 patients with high baseline pain intensity demonstrated a similar degree of mechanical hypersensitivity *in vivo* to that observed with IgG from the main donor. In comparison, IgG from 26 patients with moderate pain intensity, produced a more transient hypersensitivity, and required administration of larger doses. Although we cannot estimate the fraction of CRPS patients with sensitization-supporting IgG, our results with the pooled samples are consistent with such antibodies being found in a substantial portion of chronic CRPS patients. If a substantial proportion of CRPS patients with high pain intensity lack autoantibodies, we would have expected a sharply reduced behavioral response in experiments with pooled IgG from this group of patients. The reduced activity of IgG from patients with moderate pain severity suggest a correlation between the reported pain intensity and the effective serum concentration of autoantibodies. These conclusions are consistent with previous studies of IgG from a number of patients with longstanding severe CRPS, all of which produced mechanical hypersensitivity in the tCRPS model[31,55]. Importantly, the increased spontaneous and evoked action potential discharge rate observed here in *ex vivo* skin-nerve preparations demonstrates that patient IgG produce nociceptor hyperexcitability.

CRPS has very recently been reported to be associated with an expansion and activation of memory T-cells[47] and there is evidence of an altered profile of tissue resident cutaneous T-cells in the limb affected by CRPS[5,41]. It is not yet clear how the initial trauma contributes to the subsequent autoantibody mediated pathology in CRPS [43]. It is possible that the injury induces production of neoantigens and that the plasma extravasation, produced around the trauma at a time when the blood nerve barrier may be compromised[52], facilitates access of IgG to the affected area. Intriguingly, studies of the tibial fracture, cast immobilization experimental model of CRPS have demonstrated that mice in this model reproducibly develop long-lasting, painful sensory abnormalities and vascular dysregulation in the injured limb, very similar to the acute phase of CRPS[29,38]. This CRPS-like experimental condition is produced by IgM-mediated autoimmunity, which can be transferred between mice [29] and it is associated with reactivity against proteins that are upregulated or undergo a cellular redistribution after the fracture[54]. Clinical observations suggest that early immobilization after fractures increase the risk of CRPS in humans[13,19], and results from the tibial fracture, cast immobilization mouse model may indicate that IgM mediated autoimmunity is responsible for the onset and early phase of CRPS.

An emerging body of evidence indicates that autoantibodies are the primary pathological agents responsible for pain in some painful disorders other than CRPS [15,24]. Autoantibodies that target citrullinated proteins produce pain in rheumatoid arthritis (RA) by stimulating IL-8 release from osteoclasts, even when inflammation is clinically well-controlled [11,62]. Serum-IgG from patients suffering from rare painful neurological autoimmune disorders targeting the voltage-gated potassium channel complex (VGKCC, specifically CASPR2) elicit painful hypersensitivities upon transfer to mice[16]. CASPR2 autoantibodies cause pain by reducing the surface expression of Kv1 channels and enhancing the activity of D-hair Aδ-fibers. These earlier mechanistic investigations thus demonstrate that autoantibodies can cause pain either indirectly, by stimulating release of proalgesic mediators from other cells[11], or by directly affecting the activity of sensory neurons[16]. Importantly, VGKCC autoantibodies have not been observed in CRPS patients[4,33,42], and we did not detect functional abnormalities in D-hair fibers in tCRPS mice, highlighting that VGKCC autoantibodies generate pain by mechanisms that are distinct from those that are engaged by CRPS IgG.

The proteins and processes by which autoantibodies in persistent CRPS produce pain and hypersensitivity remain unknown, but earlier investigations have demonstrated that a subset of patients produce IgG that recognize epitopes on autonomic neurons and SH-SY5Y cells[35]. Further studies identified specific binding to M2 muscarinic as well as to β2- and α1 adrenoreceptors[18,34], but the relevance of these receptors for CRPS pain and autonomic signs is not yet clear.

Here, we identified an increased spontaneous impulse rate in the intact saphenous nerve in skin-saphenous nerve preparations from tCRPS mice. Although it is not possible to determine which fiber types are responsible for the heightened ectopic activity in our preparations, such ectopic discharge is likely to be responsible for spontaneous pain and paraesthesias[50,51,60]. We further identified an increased responsiveness to mechanical stimulation of AM- and CM nociceptors characterized both by a reduced mechanical threshold and by an increased impulse discharge rate in response to supra-maximal stimulation. It is likely that this increased peripheral drive gives rise to spinal sensitization, which would be consistent with the recent finding that spinal mechanisms involving interleukin-1β are engaged in tCRPS[31]. Upregulation of spinal interleukin-1β has previously been demonstrated in experimental models of neuropathic and arthritic pain[17,20].

The results presented here support the use of immune therapies, such as plasmapheresis[2] or B-cell ablation, to reduce autoantibody titre in patients with persistent CRPS. The variable time required for mice to recover from tCRPS suggests that patients may need to undergo relatively extended treatment periods to benefit from reduced IgG titres, consistent with the available data from trials with therapeutic plasma exchange[26].

Our results demonstrate that IgG autoantibodies from CRPS patients generate painful hypersensitivities by increasing the activity of peripheral nociceptors and this new information may guide future attempts at identifying targets for interventions.

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FIGURE LEGENDS

Fig. 1. **CRPS IgG produce polymodal hypersensitivities in mice**. Administration of CRPS patient IgG (8mg, i.p. on days -1 to 2), prolonged and exacerbated the ipsilateral mechanical (**A**), cold (**E**) and heat (**G**) hypersensitivity produced by a paw incision (on day 0) compared to mice treated with IgG from healthy control subjects or no IgG. The sensitivity to stimulation with von Frey filaments was unaffected by CRPS IgG (**C**). The contralateral, uninjured paw displayed a much less marked hypersensitivity in all four tests (**B**, **D**, **F**, **H**). Data are mean ± SEM of n=6. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA, followed by Tukey's, CRPS compared to HC IgG group. †p<0.05, ††p<0.01, †††p<0.001, One-way ANOVA, compared to naïve values before surgery and injection, Dunnett’s post-hoc test.

Fig. 2. CRPS IgG induced dose-dependent and sustained hypersensitivities. Effect of different dose regimens of serum IgG from CRPS patient compared to HC IgG on ipsilateral (A) and contralateral paw (B) withdrawal thresholds to mechanical pressure, and (C) ipsilateral and (D) contralateral paw withdrawal latencies to noxious cold (10°C). IgG was administered on days -1 to 2 (4 injections) or days -1 and 0 (2x8mg), and paw incisions were performed on day 0. Data are mean ± SEM of n=6. Data points from all groups and time points were tested by one-way ANOVA followed by Holm-Sidak's post-hoc: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs HC IgG.

Figure 3. The phenotype of tCRPS mice is related to the pain intensity of CRPS patient IgG donors. (A) Ipsilateral paw withdrawal thresholds to paw pressure before and 7 days after paw incision (4 days after the last IgG injection), in mice injected with IgG (8mg) pooled from either healthy control subjects (HC), CRPS patients with moderate pain intensities, or CRPS patients with high pain intensities. (B) Paw withdrawal threshold of mice treated with 4x16mg of IgG from HC IgG or CRPS patients with moderate pain intensities, or 4x8mg of IgG from patients with high pain intensities. n=5-6. ANOVA with Tukey's post-hoc (A, B): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs HC IgG group. One-way ANOVA, pre and post-surgery comparison using Dunnett's post-hoc test (A). ††† $p < 0.001$.

Figure 4. CRPS IgG increases the spontaneous impulse rate in skin-saphenous nerve preparations. (A) Typical examples of spontaneous activity recorded in skin-nerve preparations from naïve mice, mice only subjected to a paw incision, or subjected to a paw incision in combination with IgG from HC or CRPS patient. (B) Frequency of spontaneous activity recorded in the intact saphenous nerve. Each data point represents one preparation/animal. **$p < 0.01$, Kruskal-Wallis.

Figure 5. CRPS IgG sensitizes AM fibers. (A) Mechanical response thresholds of AM fibers in preparations from tCRPS mice and mice that only underwent incision. (B) Example traces of AM fiber action potentials evoked by a 5g force step and (C) a force ramp stimulus to 10g. The temporal impulse pattern is displayed in histograms (*lower panel*). (D-E) The mean number of action potentials (AP) evoked by force step (D) and ramp (E) stimuli (0.5g-20g) in preparations from incision only compared to tCRPS mice; the linear regression of the force-impulse relationship with 95% confidence interval is shown in shaded pink/gray. (F) The mean impulse pattern of AM fibers during 10s constant 5g force simulation and (G) 15s ascending force of 10g (means calculated from all fibers presented in D, E). (H) Peak firing frequency (events/s) in incision compared to CRPS groups for 5g ramp stimulation and (I) 10g ramp stimulation. Data are mean±SEM or individual data points. Levene's test $p<0.05$; Mann-Whitney U-test: *$p < 0.05$, **$p < 0.01$, CRPS group comparison with incision group.

Fig. 6. CRPS IgG sensitizes CM fibres. (A) Mechanical response thresholds of CM fibers in preparations from tCRPS mice and mice that only underwent incision. (B-C) Example traces of C-fiber action potentials evoked by a 20g force step (B), and a force ramp (C) stimulus to 20g. The temporal impulse discharge frequencies are shown as histograms (events/s). (D-E) Mean number of action potentials (AP) evoked by (D) step stimuli of (0.5g-20g) and (E) ramp stimuli in incision and CRPS mice. The force-impulse relationship is indicated by linear regression, with the 95% confidence interval shaded. (F-G) Mean pattern of action potential rate in CM-fibers in response to (F) a 10s constant 20g force application and (G) a 15s force ramp challenge to 20g. (H-I) Peak firing frequency (events/s) in incision versus CRPS groups for 20g force step (H) and ramp (I) stimuli. Data are mean±SEM. *$p < 0.05$, ***$p<0.001$, t-test (A, B, H, I) or Mann-Whitney (following Levene's test $p<0.05$), CRPS group comparison with incision group.

Fig. 7. Low threshold mechanosensitive Aβ and DH fibers are unaffected by CRPS IgG. Mechanical response thresholds determined by 2s force step challenges (A, C, E) and mean number of action potentials (AP) evoked by 10s, 0.5g-20g force steps (B, D, F) in preparations from tCRPS mice and mice that were subjected to paw incision alone. Slowly adapting Aβ fibers (A, B), rapidly adapting Aβ fibers (C, D) and DH fibers (E, F). Data are mean ± SEM. Mann-Whitney (following Levene's test $p<0.05$), tCRPS compared to incision only.

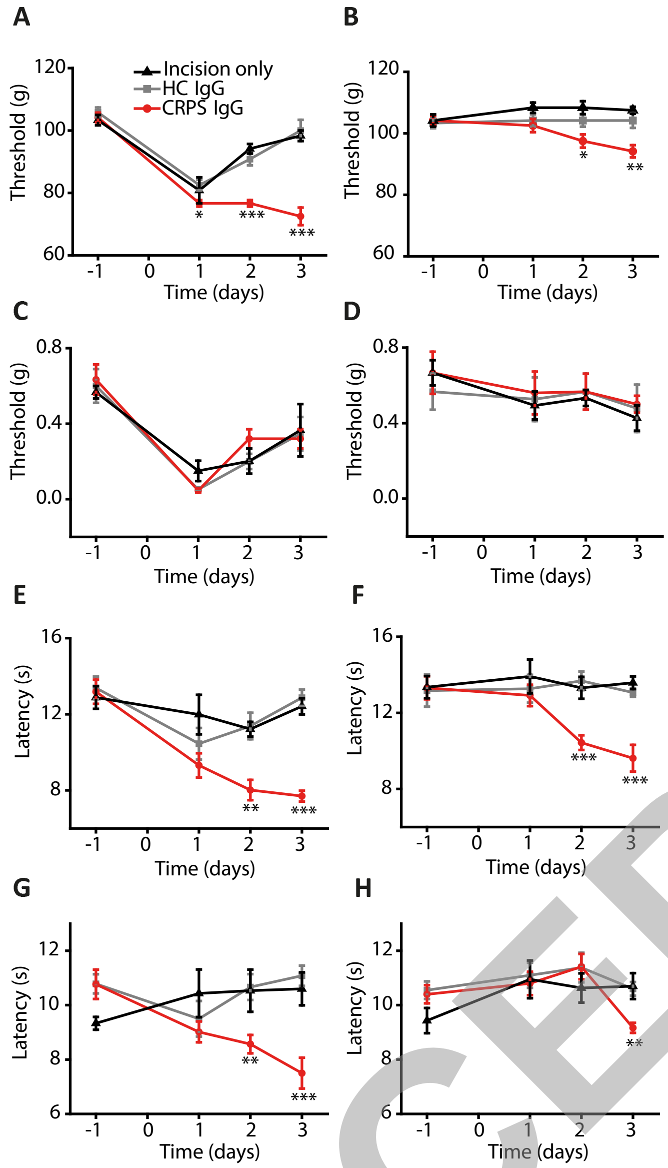
Table 1. Characteristics of patient IgG donors.

	Moderate pain (n=26)	**High pain (n=27)**
Age (years, average±SD)	43±14	41±10
CRPS duration (years, median (range))	3 (1-5)	3 (1-5)
% female	54%	77%
Baseline pain intensity (average)	6.0 ±0.8 (SD, range 5.0-6.9)	8.3 ±0.4 (SD, range 7.3-9.5)

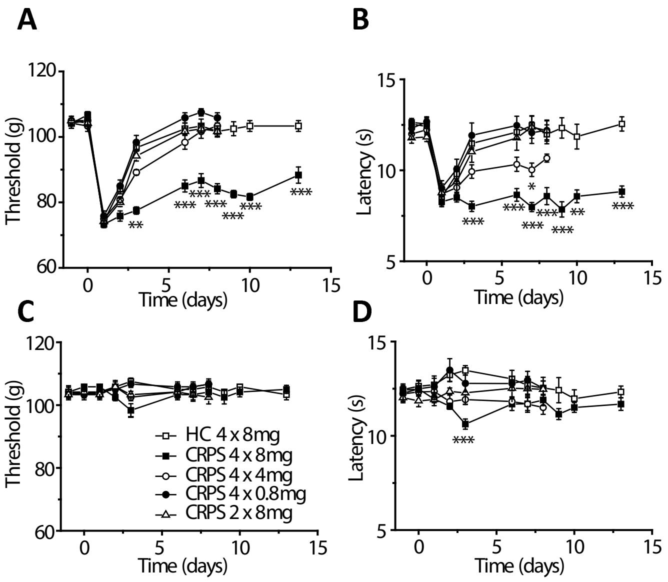
Table 2. Conduction velocity of the different fibers from incision only and tCRPS preparations. Data are mean ± SEM. AM, CM and RA-Aβ units were tested with Mann-Whitney, whereas SA-Aβ and D-hair were tested with a t-test.

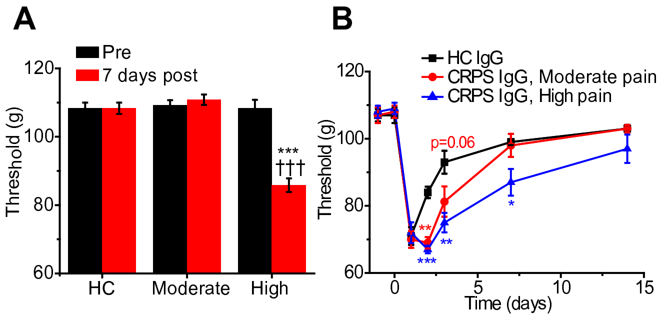
Fiber type	Incision only conduction velocity (m/s)	tCRPS conduction velocity (m/s)	P values
RA-Aβ	12. 0±0.6 (n=16)	15.7±2.0 (n=12)	0.07
SA-Aβ	12.9±0.7 (n=14)	13.2±0.5 (n=13)	0.85
D-hair	6.3±0.7 (n=10)	7.2±0.8 (n=9)	0.39
AM[1]	7.3±0.7 (n=17)	6.2±0.6 (n=23)	0.28
CM[2]	0.52±0.1 (n=14)	0.78±0.1 (n=13)	0.07

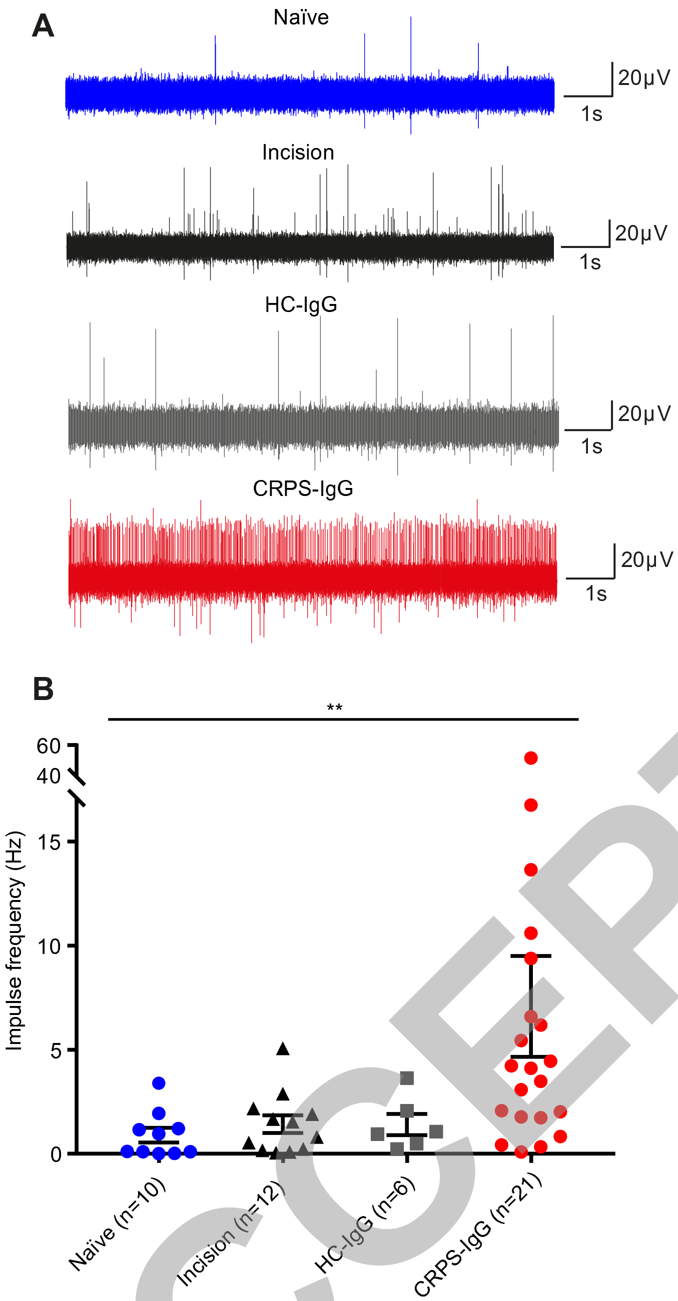
[1]*Aδ- mechanonociceptor,* [2]*C-mechanonociceptor*

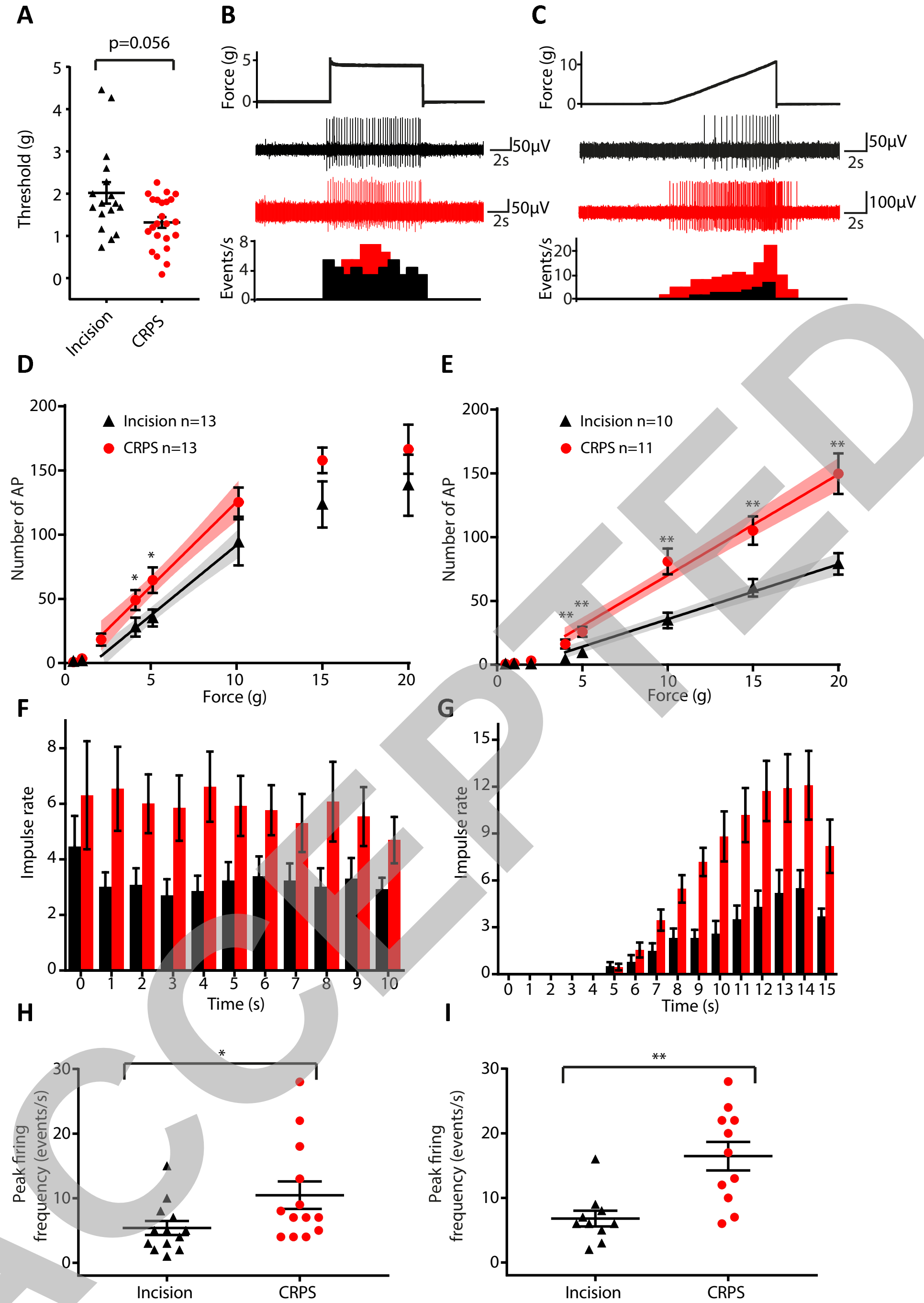


A
B
C
D
E
F
G
H
Incision only
HC IgG
CRPS IgG
Threshold (g)
Latency (s)
Time (days)

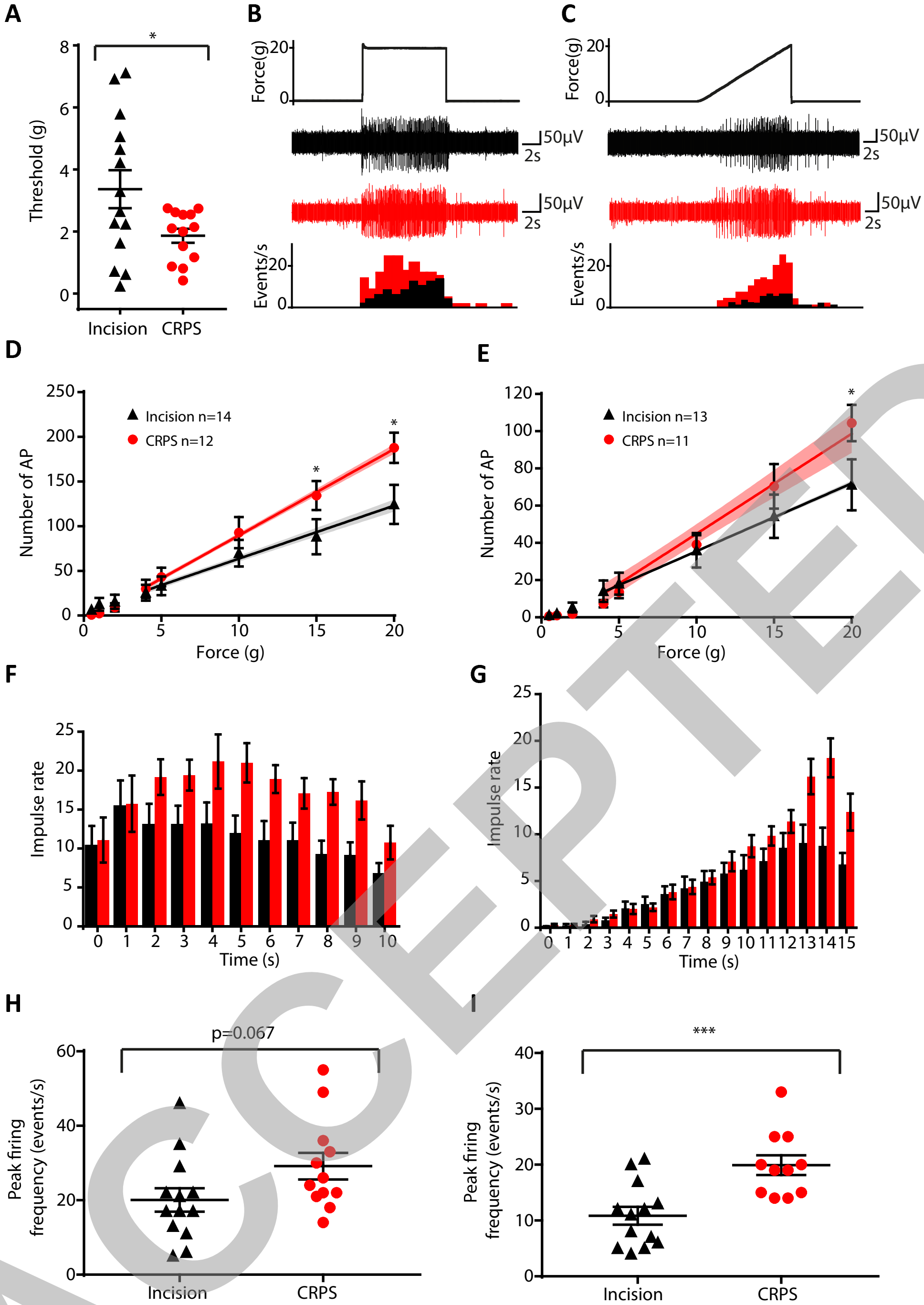

A
B
C
D
Threshold (g)
Latency (s)
Time (days)
HC 4 x 8mg
CRPS 4 x 8mg
CRPS 4 x 4mg
CRPS 4 x 0.8mg
CRPS 2 x 8mg

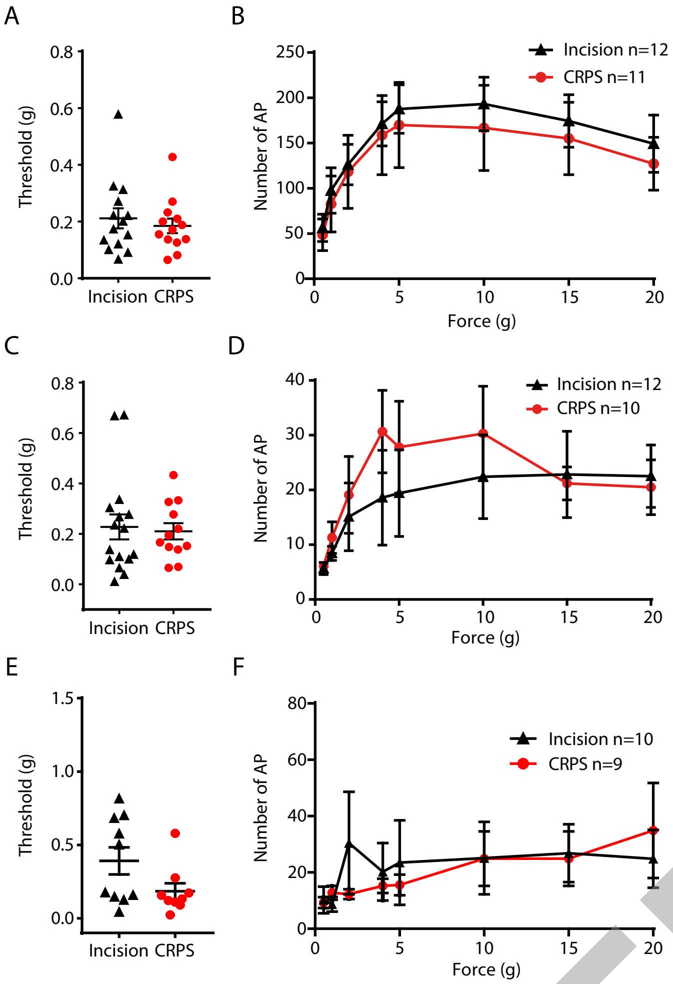

A
Pre
7 days post
120
100
80
60
Threshold (g)
***
†††
HC
Moderate
High
B
HC IgG
CRPS IgG, Moderate pain
CRPS IgG, High pain
120
100
80
60
Threshold (g)
p=0.06
**
***
**
*
0
5
10
15
Time (days)


A
Naïve
20μV
1s
Incision
20μV
1s
HC-IgG
20μV
1s
CRPS-IgG
20μV
1s
B
**
60
40
15
10
5
0
Impulse frequency (Hz)
Naïve (n=10)
Incision (n=12)
HC-IgG (n=6)
CRPS-IgG (n=21)



A
B
C
D
E
F
G
H
I
p=0.056
Threshold (g)
Incision
CRPS
Force (g)
Events/s
50µV
100µV
2s
Incision n=13
CRPS n=13
Incision n=10
CRPS n=11
Number of AP
Force (g)
Impulse rate
Time (s)
Peak firing frequency (events/s)


A
B
C
*
Threshold (g)
Incision
CRPS
Force(g)
Events/s
50μV
2s
D
E
Number of AP
Force (g)
Incision n=14
CRPS n=12
Incision n=13
CRPS n=11
F
G
Impulse rate
Time (s)
H
I
p=0.067
***
Peak firing frequency (events/s)



A
B
C
D
E
F
Threshold (g)
Incision
CRPS
Number of AP
Force (g)
Incision n=12
CRPS n=11
CRPS n=10
Incision n=10
CRPS n=9